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Reviewer's Report on Doctoral Thesis

"Influence of functionalized nanoparticles of different sizes, material, and surface properties on cellular machinery"

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The Doctoral Thesis of Mariia Uzhytchak is focused on *one of the key problems* of nanomedicine, exploiting inorganic and organic nanoparticles (NPs) as agents for drug delivery, cancer diagnostics, and therapy. Although some nanoparticles are already used in clinical applications and preclinical studies, this field is relatively young and many questions are still unresolved concerning mechanisms of NPs penetration into living cells, and their impact on cellular machinery, metabolism, and cytotoxicity. The lack of knowledge hampers efforts in using functional nanoobjects in their safe and efficient use for preventing, improving diagnostics, and treating cancer diseases. By present, different types of NPs are studied as drug delivery and/or anticancer agents. The Thesis focuses on the study of the interaction of iron oxide nanoparticles (IONPs) and DNA nanostructures (DNs) with living cells. Three distinct cell lines have been used in experiments, Huh7 cells, Alexander (PLC/PRF/5) cells, and the human hepatoblastoma cell line HepG2. A comprehensive strategy of versatile diagnostic techniques has been elaborated in this study that allowed to introduce *a substantial novelty* into understanding the basic mechanisms of how NPs are uptaken in cells and what are their influence on metabolism as well as their cytotoxicity effect. Important is that, in the investigations, comparisons were made for two types of NPs, IONPs and DNs, which are considered as highly promising objects for nanomedicine. Understanding of pros and cons for their utilization in diagnostics and treatment of cancer with possible harsh side effects is of vital need for present state-of-the-art of nanomedicine.

The Thesis is very well structured into chapters, subchapters, and appendices, which represent the publications co-authored by Mariia Uzhytchak with indications of her significant role in obtaining the published results. The first Chapter "Introduction" presents a detailed review of the state-of-the-art in the field of nanomedicine connected with utilizing different kinds of nanoparticles, both inorganic and organic, as drug delivery, diagnostic, and therapy agents. The Chapter introduces definitions used in nanomedicine, gives an overview of main developments, describes in detail the physicochemical properties and potential in biomedical applications for IONPs and DNs as the main objects of the Thesis research, and outlines existing problems and challenges as well as prospective for further research and application. This part of the Thesis is written vividly in very good language and contains enough scientific details but also can be understandable by a wider public. It demonstrates a deep understanding by the candidate of the specific field of research, her broad outlook on science and technology, and her ability for popularization of scientific achievements. In the Introduction chapter, 131 articles are cited, thus giving an impressive overview of the present status of research and justifying the topic of the Thesis work as one in the frontier in nanomedicine.

In the Second Chapter, the aims of the study are stated concisely and very clearly. It was declared that IONPs are used as a model of current nano-drugs utilized in several applications, including MRI, magnetic drug delivery, and hyperthermia while DNA nanostructures are investigated as a novel and promising nanomaterial. The central goals are to reveal how nanoparticles are processed within the cells (their transport mechanisms, association with organelles, and degradation) and, what is important, is to achieve

an understanding how the cells are responding to nanoparticle incorporation. The following chapters demonstrate that the aims of the PhD work have been achieved in full.

The Third Chapter summarizes in detail the materials and methods used in the PhD study. It is important that the study has not been focused on one cell type from one company as different cell types can differently respond to nanoparticles and vital is to apply a versatile framework to reveal the specificity of cells' reaction. Three types of cells have been studied, Huh7 cells from the Japanese Collection of Research Bioresources, Alexander (PLC/PRF/5) cells from the American-Type Culture Collection, and the human hepatoblastoma cell line HepG2. Determining the optimal growth conditions of cell cultures and the methodology of protein extraction have been described. To investigate cellular features, several microscopy techniques were utilized, an IM-2FL epi-fluorescent system, a Nikon Diaphot 200 microscope, and an IXplore SpinSR system, that enabled the comprehensive analysis of apoptosis and exceptionally detailed cell visualization. For measuring absorbance, luminescence, or fluorescence signals from a larger number of samples, the Tecan microplate reader SpectraFluorPlus was utilized. The application of multifaceted analysis techniques secured high-quality and reliable results described in the next Chapter.

Chapter 4 is devoted to a detailed description and discussion of the obtained results which are rich and lead to identification of the further directions for essential studies in the field. Here I list only several *new observations* and the *most important results* reported by Mariia Uzhytchak:

- The obtained data revealed a concentration-dependent effect when higher IONPs concentrations led to increasing NPs uptake by the cells. Additionally, application of pulsed magnetic fields resulted in an additional enhancement in IONPs uptake. These studies indicated that applying intense pulsed magnetic fields did not have toxic effects on the cells and can be used in practice to facilitate IONPs uptake by cells.
- It was found that the pulsed magnetic field (PMF) applied on superparamagnetic IONPs clusters considerably influences cell death. The mechanism of apoptosis of superparamagnetic IONPs-loaded cells treated with PMF was identified as mitochondrial membrane permeabilization and dysfunction. This mechanism was supported further by using JC-1 dye to assess mitochondrial membrane potential.
- The studies of cellular interactions of IONPs have shown that they may cause a delayed cytotoxic effect in different cell types, primarily through the excessive production of reactive oxygen species. Furthermore, it was found that IONPs accumulation takes place within lysosomes that results in change of lysosomal size and shape and thus affects lysosomal functionality.
- It was demonstrated that treatment with NPs differently influences the cells under study, revealing no change in the activity of mTOR protein in HepG2 cells while a decrease in nuclear mTOR phosphorylation in Alexander cells and an increase in cytosolic mTOR activity in Huh7 cells was detected. This points out that a cell-specific response to NP treatment has the potential to be used for cancer prognosis.
- An original investigation of DNA nanostructures (DNs) interaction with living cells has been performed. Uptake kinetics of DNs by Alexander, HepG2, and Huh7 cell lines were studied revealing substantial uptake difference and the significant role of cell size in the uptake process. The obtained data have also revealed a specific pattern of endosomal escape of the coated DNA nanostructures (EE-DNs) uptake. As a whole, these results underscore the significant role of cell size in the uptake of DNs and the stability of these nanocarriers within lysosomal compartments.

In the Conclusion section, a comprehensive analysis of the main achieved results and their significance in decreasing the drawback of knowledge in the field of nanomedicine are summarized. In particular, it is underlined that hepatic accumulation of IONPs, which were usually described as biocompatible, and their

interaction with liver cells have revealed a complex interplay of biological processes that may lead to considerable manifestation of hepatotoxicity.

As a whole, the obtained results of comparative investigations of the impact of inorganic and organic nanoparticles on cancer cells and living organisms as a whole and their analysis performed in the frames of the doctoral work are of valuable fundamental and practical importance as they demonstrate new insights into the effects of NPs in nanomedical applications. The performed work gives indications to the perspectives and the possible routes for potential treatments of cancer by means of nanomedicine. Still limited data in this field call for further investigations in this multidisciplinary field that should include pharmaceutical sciences, cell biology, engineering, chemistry, and materials science. The thesis is very well written and negative remarks or questions did not appear during reading.

The main results of the Thesis have been reported in 8 articles published in indexed peer-reviewed journals, several conference contributions, and lectures. Summarizing, the candidate for PhD degree has performed large amount of insightful research and obtained new original results, which broaden our understanding in applications of inorganic and organic nanoparticles as potential agents for drug delivery and cancer diagnostics and treatment. The doctoral work has been performed at a high scientific level. All important results presented in the Doctoral Thesis have been published in peer-reviewed journals. Judging by the Dissertation, the candidate Mariia Uzhytchak merits the PhD Degree.

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